Mutualistic symbiosis of algæ and bacteria with Cycas revoluta.

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WITH PLATES III AND IV.

Recently my attention was directed to the tubercle-like growths on roots of *Cycas revoluta.* A cursory examination showed that they were infested by a nostoc. In my search for the literature on the subject I found few and incomplete references. Between 1870 and 1873 Reinke discovered parasitic Nostocaceæ in species of Gunnera and Cycas. Janczewski discovered parasitic algæ in mosses, Cohn in Lemna, Kny in Florideæ and Strasburger in Azolla.

Reinke is to my knowledge the only person calling attention to an Anabaena found parasitic in a specialized parenchyma layer of Cycas roots. His incomplete though exact description has induced me to study the subject more closely.

Cycas root tubercles, which are simply short somewhat enlarged dichotomously branched rootlets, are quite common on most of our cultivated cycads. They occur on young as well as on old plants. The youngest plants at my disposal were about two years old. Only a few tubercles were present. A large, well nourished plant about twenty-four years old had many tubercles. They were most numerous near the surface of the soil; a few were wholly above and some were found a foot or more below the surface. Usually they are formed from the ends of rootlets, sometimes from the side of root branches, especially the single unbranched tubercles. In position they show evidence of negative geotropism. This is very marked in tubercles near the surface of the soil. Branching is always dichotomous (see plate III, fig. 3). Branches are short and somewhat spindle-shaped, the ends being bluntly rounded. Why they should branch dichotomously is interesting. It is probably a form of atavism showing the relation of cycas to the vascular cryptogams. Likewise the occasional dichotomous branching of leguminous tubercles may indicate a descent from cryptogams.[1] As to color one may readily distinguish

[1]It may be mentioned here that the relative positions of the phloem and xylem in the vascular system of leguminous tubercles corresponds to that in Cycas.

three kinds of tubercles. Those of a yellowish tan color, generally found above the surface, are devoid of the symbiotic algæ; the second variety, of a slightly darker tan and often greenish near the tip, always contain the algæ; they are the younger tubercles. The third variety, which also contain the algæ, are of a dark brown color and are older than the others. These three varieties are found on the same plant. In external appearance they resemble somewhat the tubercles of *Pisum sativum.* Morphologically they differ considerably. In pea tubercles the symbionts are surrounded by the vascular bundles while in Cycas the symbionts surround the centrally located vascular system. They resemble each other in their mode of branching.

Their mode of development is quite simple. Either the ends of rootlets branch dichotomously or they develop endogenously. It may be more correct to say that all tubercles develop like primary roots and that the lateral development is only apparent. That is the developing lateral root branch receives its tubercular peculiarity from the very start. The line of demarcation between rootlet and tubercle is very distinct and abrupt. The tubercle branch has about three or four times the dimensions of a rootlet of the same length.

On making a cross section of any part of the tubercle excepting the tip one can see with the naked eye a green circular layer about midway between the epidermis and bundle sheath. This is the alga-bearing layer. At certain points this green layer is discontinuous. This always occurs opposite outer lenticular structures which are quite common on the tubercles and are arranged in more or less broken rings. Having thus treated of the gross anatomy I shall next describe the minute anatomy.

A cross section shows six tissue layers. The first and outermost is the dermal layer of irregular corky cells several rows in thickness developed from a dermatogenic layer dividing tangentially. In the dermal layer are also included the lenticular structures consisting of enlarged irregular corky cells which do not seem to develop from any definite phellogenic layer. The cell walls of the dermal layer give to the tubercle its yellowish or dark brown color. The cells contain, besides the remains of nuclei and cytoplasm, various kinds of rhizobia in comparatively small numbers. The entire surface of rootlets, roots and tubercles is more or less covered by rhizobia, bacteria, hyphal fungi and various species of algæ, the discussion of which will be taken up below. The second layer is the dermatogenic layer of tangentially elongated rectangular thin walled cells. The third layer is the subdermal parenchyma of large rounded cells with intercellular spaces and air conducting passages. Considerable starch is present and often oil globules are found toward the inner side where the most rhizobia and bacteria are also found. The fourth layer is of the most importance. It consists of two opposite rows of palisade cells. This layer is only present in tubercles bearing algæ and is formed from radially elongating parenchyma cells beginning near the apical area of the tubercle and extending near the point of separation between tubercle and rootlet. The two rows of palisade cells are separate or only loosely connected in the middle. The cells are thin walled, about two or three times as long as broad, with large nuclei suspended in a granular cytoplasm. The large intercellular spaces are entirely filled with algæ (*Nostoc* sp.?). Besides the granular cytoplasm, the cells contain starch, amyloplastids, sometimes oil globules, and a waxy body near the base. The fifth layer or parenchyma proper resembles the subdermal parenchyma. The cells contain much starch. Numerous cells entirely filled with a waxy substance are present. The sixth layer is the vascular system sheath consisting of modified parenchyma cells of several layers thickness. The vascular system need not be described as it is the same as that of the ordinary root.

A longitudinal section shows the presence of a rudimentary root cap consisting of elongated loosely connected cells covering more or less perfectly the rounded end of the tubercle. The apical area consists of small prismatic closely united meristem cells. The palisade cells do not extend quite to the apex. (See plate III, fig. 2.)

The exact cause of the development of these tubercles I am unable to state. That there is excessive metabolism is very evident from their appearance and the large amount of albuminous substances present. That the infecting algæ are not the cause of their development is shown by the fact that tubercles exist without the alga-bearing palisade layer. On making a comparative study of Cycas roots and tubercles I found the following differences: The dermal and apical area of tubercles contained more rhizobia and bacteria than similar structures in ordinary roots and rootlets. Also the cytoplasm of tubercular cells is more granular, that is, it contains larger and more prominent dermatosomes, especially the palisade cells. It is very likely that whenever a certain amount of rhizobia and bacteria have infected the apical area of a certain rootlet, their irritating presence produces increased metabolism and rapid branching. Thus the increased number and size of the dermatosomes would be due to the rhizobia.

The question of the symbiotic relation of Cycas, algæ and rhizobia is very interesting and apparently rather complex. All soils, especially green house soils, contain rhizobia and other bacteria besides numerous algæ. These algæ are very common both in the soil and the vessels in which green house plants are grown. That they play an important part in binding the free nitrogen of the air has been conclusively shown by B. Frank. In examining carpellary and the rudimentary hypsophyllary leaves of Cycas it was seen that they often had a greenish coating consisting of algæ. The following genera were noted: Protococcus, Navicula, Chroococcus, Oscillaria, Glœocapsa, Ulothrix, Chlœosporium and Nostoc. Numerous rhizobia, bacteria and several species of hyphal fungi were also found. Among the rhizobia I could readily recognize *Rhizobium mutabile*,[2] *Rhizobium curvum*, and *Rhizobium Frankii* beside many, to me unknown, species of bacteria and cocci. Examination of the surface soil in which the plant grew showed a similar protophytic flora though in somewhat lesser abundance. The predominating types among the algæ seemed to be Protococcus and Nostoc. Among the rhizobia and bacteria I could find no predominating type.

Cross sections of tubercles showed that no algæ are inside the cells, while nearly all of them, especially those of the dermal layer, contained more or less rhizobia and bacteria. Parenchyma cells and bast cells of vascular system contained some rhizobia and bacteria. They seemed to be quite abundant in the apical area. Culture experiments developed three predominating types; a coccus, a probable rhizobium resemblimg *Rhizobium Frankii*, and a larger Indian club shaped bacterium resembling somewhat *Rhizobium mutabile* of *Trifolium repens* though smaller and of a more constant size and form (plate IV, figs. 6, 8).

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[2]Bull. Torr. Bot. Club. **19**: 203. July, 1892.

Culture experiments were made with special precautions to prevent the introduction of bacteria, etc., everywhere present on the surface of both tubercles and roots. As a suitable medium a slightly acid agar-vegetable root extract was prepared. Tubercles of normal appearance were secured and carefully and thoroughly washed with plenty of hydrant water then quickly dried by means of blotting paper which had been passed through the flame of a Bunsen burner. A tangential section, including somewhat more than the dermal layer, was made down one side of the tubercle, then the tubercle was passed through the flame of the Bunsen burner so as to singe it thoroughly on all sides, and then broken (not cut) across. Inoculations were made from the broken surface farthest from the cut side. The inoculated tubes were placed in a dark chamber at the ordinary summer temperature (Ills.). In about six or seven days a small whitish growth was noticed in most tubes. Cultures made from the dermal and hypodermal parenchyma generally developed organisms resembling *Rhizobium Frankii* (plate IV, fig. 6), and I shall provisionally place them with that genus. Inoculations made from the palisade layer sometimes developed a coccus, more often there was no growth at all. As a rule there are no bacteria or cocci to be found with the infecting nostoc. Cultures made from the vascular system, especially near the apical area, generally developed the above mentioned *Rhizobium*, but more often a peculiar Indian club shaped organism (plate IV, fig. 8). I was unable to obtain absolutely pure cultures, but the two forms of bacteria (or rhizobia) described seemed to predominate. Cultures in which the rhizobium-like organism predominated finally took on a yellowish color. In this respect it resembled very much cultures of the rhizobia from the "Infektionsfäden" of *Melilotus alba* or *Trifolium pratense*. This organism resembles in appearance *Rhizobium Frankii* of *Phaseolus vulgaris* but differs in that it has cilia and is motile during its earlier life history.[3] Rhizobia and bacteria are not present in large numbers in any part of the tubercle. The question whether their presence is purely accidental or whether they live in active mutualistic symbiosis with Cycas could not be determined in the short time at my disposal. It is however quite certain that tubercles contain more bacteria

[3]Further experiments in regard to the determination of the rhizobia are now in progress.

and rhizobia than the normal root. It is also certain that there is greater cytoplasmic activity in tubercles than in the normal roots; this is shown by the greater abundance of albuminous substances present and the greater prominence of the cytoplasmic granules[4] (Dermatosomen, Plasomen, etc.) (plate IV, fig. 7).

As already stated the dark tan and dark brown tubercles always contain the infecting nostoc. It is generally taught that algæ can not develop in the dark. This is evidently not true as some of the nostoc-bearing tubercles are found as much as a foot below the surface of the soil. In fact tubercles wholly above the ground never contain the nostoc. That the nostoc is the cause of the development of the palisade layer is quite evident from their constant association.

The exact mode of infection is as yet undetermined. The nostoc no doubt enters the parenchymatous tissue of the tubercle through a break in the dermal layer soon after it begins to form. Why the nostoc should take up a definite position in the parenchyma midway between the dermal layer and vascular system sheath is as yet unexplained. The parenchyma cells nearest the nostoc appropriate the extra nitrogenous compounds stored by the infecting symbiont, this produces hypernutrition of the incipient palisade cells which elongate in a direction parallel to the easiest conductivity of nutritious substances, that is practically at right angles to the vascular system. They serve a similar function to palisade tissues in other positions, as in leaves. Nostoc, so to speak, takes the place and serves the function of chloroplastids in true palisade cells.

Nostoc is the only alga found in the tubercles. This is probably because it is more closely related to Schizomycetes than Protococcus or Ulothrix. It is therefore better adapted to lead a parasitic or a symbiotic existence. From a study of the infecting alga I conclude that it is *Nostoc commune*. Reinke placed it with Anabæna since he could detect no gelatinous imbedding material. I found however that they were quite firmly united to each other and to the palisade cells by a gelatinous substance. Cells are spherical, loosely united, forming longer or shorter strings. Division takes place at right angle to nostoc chain. Sometimes a cell divides parallel

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[4] Contribution to the probable biology of Plasomen, Bull. Torr. Bot. Club **20**: 339. Oct. 1893.

to nostoc filament thus producing a new filament at right angles to the original. I could detect no difference in color and general behavior from nonparasitic nostoc cells. Heterocysts increase in number with the age of the tubercle. Sometimes, in very dark colored tubercles, heterocysts are present in greater numbers than the normal cells. I could detect no spore formation.

From the appearance of the host it seems quite evident that the infecting symbionts are far from harmful. The omnipresence and importance of schizophytic organisms in and on tissues of vascular cryptogams and gymnosperms is probably far from being overestimated.

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Explanation of Plates III and IV.

Plate III.—Fig. 1. Portion of cross section of *Cycas revoluta* tubercle representing dermal (lenticular), dermatogenic, subdermal parenchyma, palisade, and parenchyma layers. Nostoc *in situ.*

Fig. 2. Portion of longitudinal section of tubercle showing apical area without the root cap. Beginning of palisade layer with a few nostoc filaments.

Fig. 3. Diagramatic section of tubercle, showing manner of branching and distribution of vascular bundles.

Fig. 4. *Nostoc commune.*

Plate IV.—Fig. 5. Some of the parenchyma and palisade cells highly magnified showing reticulated structure of cytoplasm and nucleoplasm. Larger black dots in cells represent full grown plasomes or dermatosomes. Starch and resinous bodies in some of the palisade cells.

Fig. 6. *Rhizobium* sp. ? from dermal layer.

Fig. 7. Plasomes from palisade cells.

Fig. 8. Bacteria from parenchyma and vascular tissue

(Figs. 6, 7 and 8 very highly magnified.)